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ROLE OF BACTERIAL PECTIC AND PROTEOLYTIC ENZYMES IN POSTHARVEST DISEASES OF FRUITS AND VEGETABLES

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ABSTRACT

Pectolytic fluorescent pseudomonads, mainly strains of *Pseudomonas fluorescens* and *P. viridiflava*, are the cause of substantial proportions of postharvest losses on an array of horticultural crops including fruits and vegetables. The ability of these pseudomonads to cause disease (often in the form of soft-rot) results primarily from their ability to degrade plant cell walls by producing two depolymerizing enzymes, pectate lyase (Pel) and protease (Prt). Unlike the multiple Pel isozymes demonstrated in *Erwinia* spp., pectolytic fluorescent pseudomonads produce a single Pel with an approximate pI of 9.7-10.0 and an approximate molecular weight of 41-42 KDa. Some biochemical properties of Pel purified from *P. fluorescens* and from *P. viridiflava* have been determined. These properties include Km, Vmax, and optimal pH, temperature, and ion requirements for catalytic activity. The Prts produced by both pseudomonads are heat-resistant and dependent on zinc for activity. A set of bacterial genes (*pel, prt, out*, and *rep*) involved in the synthesis and export of Pel and Prt have been identified by transposon mutagenesis; and some of these have been cloned and further characterized. Molecular genetic analysis of these genes indicates that: (a) the Pel is the sole or principal enzyme responsible for tissue-maceration caused by soft-rotting pseudomonads; (b) both Pel and Prt contain a putative signal peptide and are excreted into extracellular milieu through common secretory machinery (Out) in the bacterium; (c) production of extracellular enzymes and exopolysaccharide (alginate) is under the control of a positive regulator *rep*; and (d) presence of calcium is absolutely required by the bacterium to produce Pel and Prt, and to cause disease in plants. These studies demonstrate the feasibility of using ion chelators to control disease and the possibility of using *Pseudomonas* as a host system to produce high-valued proteins or enzymes for pharmaceutical and biotechnological applications.

INTRODUCTION

Between 10 and 30% of fresh fruits and vegetables produced in the United States are lost after they are harvested. A large part of these losses are due to diseases caused by various types of microorganisms including bacteria and fungi. The disease caused by pectolytic bacteria, often referred to as bacterial soft-rot, is

the most destructive and the most often-found decay on an array of horticultural commodities including potatoes, tomatoes, lettuce and peppers. Previously, it was assumed that *Erwinia* species were responsible for most of the decays. A study conducted at the Eastern Regional Research Center in 1987 indicated that pectolytic bacteria in diverse genera including *Erwinia*, *Peudomonas*, *Xanthomonas*, *Cytophaga*, *Bacillus* and *Clostridium* are involved. Moreover, pectolytic fluorescent pseudomonads, mainly strains of *Pseudomonas fluorescens* and *Pseudomonas viridiflava*, accounted for over 40% of the decays examined (7). These pseudomonads are nutritionally versatile and unique in that they are able to grow and cause soft-rot under refrigerated conditions. For fruits and vegetables that are stored at low temperatures, pectolytic fluorescent pseudomonads may cause more damage than *Erwinia*. During the past few years, our research has focused on the enzymatic and molecular genetic mechanism by which soft-rotting pseudomonads induce diseases in plants. In this presentation, some unique features about the pectic and proteolytic enzyme system of soft-rotting *Pseudomonas* will be described.

THE PECTIC ENZYME SYSTEM OF *ERWINIA*

Much of our current knowledge about the pectic enzymes has been obtained from the studies of *Erwinia* (3). Soft-rotting erwinias produce four distinct types of pectinases, including pectin methylesterase, polygalacturonase, pectin lyase, and pectate lyase. Each of these pectinases degrades a preferred pectic substrate (pectin or pectate) at a specific site either by hydrolytic or *trans*-eliminative action, or by saponification (Table 1). Although the role of each individual pectinase in disease development is not clear, the pectate lyase (Pel) is generally believed to be the principal enzyme responsible for tissue-maceration. Another interesting feature about the *Erwinia* Pel is that this enzyme always exists in three to five isozymic forms both in cultures and in plants. At present, the pathological function of each individual isozyme is also not clear. However, it has been repeatedly demonstrated that the isozyme with an alkaline pI (9.5 or higher) is much more efficient in inducing tissue-maceration than those with neutral or acidic pIs. The biochemical basis for the difference in tissue-macerating ability among Pel isozymes has not yet been determined.

BIOCHEMICAL CHARACTERIZATION OF PECTIC ENZYMES FROM *PSEUDOMONAS*

The pectic enzyme system of soft-rotting pseudomonads is, in general, much simpler than that of erwinias. So far, only a few strains of *P.fluorescens* and *P. viridiflava* strains have been shown to produce pectin methylesterase, polygalacturonase, or pectin lyase. However, all but one strain of soft-rotting pseudomonads so far examined have been shown to produce pectate lyase (Pel)

In order to determine if *Pseudomonas* Pel also exists in multiple isozymic forms similar to those demonstrated in *Erwinia*, we employed isoelectric-focusing (IEF) and overlay activity-stain techniques to analyze the Pels produced by various strains of pseudomonads (4). All eight strains of *P. viridiflava* examined were found to produce a single Pel with an approximate pI of 9.7. And all ten strains of *P. fluorescens* examined were also found to produce a Pel with an appproximate pI of 10.0. Production of a single alkaline Pel therefore represents a common feature among soft-rotting pseudomonads.

Due to the simplicity of the enzyme system and the extracellular location of the enzyme, the Pels of *P. fluorescens* and *P. viridiflava* were easily purified from culture filtrates by two simple steps including ammonium-sulfate precipitation and ion exchange chromatography (4,8). Analysis of enzyme samples by SDS-polyacrylamide gel electrophoresis revealed that the Pels of *P. fluorescens* and *P. viridiflava* had been purified to near homogeneity. The molecular weights (Mr) of the Pels from *P. fluorescens* and from *P. viridiflava* were estimated to be 41 and 42 KDa, respectively. Analysis of the purified samples by IEF further confirms the presence of a single Pel and the alkaline nature of the enzymes (4,8).

In addition to Mr and pI, the Pels from *P. fluorescens* and *P. viridiflava* are slightly different in other biochemical properties including Km, Vmax, and optimal pH and temperature for activity (Table 2). For example, the optimal temperature for *P. fluorescens* Pel is 46°C: whereas the optimal temperature for *P. viridiflava* Pel is 52°C. Calcium is absolutely required by both Pels for activity, and the activity is inhibited by EDTA. The optimal calcium concentration for activity is in the range of 0.3 to 0.8 mM. In addition, the heat stability of both Pels is greatly enhanced in the presence of positively-charged molecules such as calcium or polylysine, but reduced in the presence of negatively-charged molecules such as polygalacturonate or heparin (15). More importantly, it should be noted that pure Pel samples in microgram quantities are capable of causing complete hydrolysis of potato cell walls and total disintegration of potato tuber disks (14). This result provides further evidence that the Pel is the sole enzyme required for tissue-maceration.

ISOLATION OF MUTANTS DEFICIENT IN ENZYME PRODUCTION

Recently, the mechanism as to how the bacterium translocates proteins across the inner and outer membranes has been the subject of extensive investigations in a number of laboratories around the world (13). A simple two-step model for exoprotein secretion in Gram-negative bacteria is illustrated in Fig.1. At the Eastern Regional Research Center, we are especially interested in the molecular mechanism by which *P. viridiflava* produces and secretes Pel into culture fluids. Using transposon mutagenesis, we have identified four distinct classes of mutants that are deficient in production of Pel, protease (Prt), and exopolysaccharide (Alg) (Table 3). The Pel mutant showing negative reaction in

pectolytic but positive in proteolytic assays resulted evidently from the insertion of Tn5 into the *pel* gene. The Prt mutant, on the other hand, showing negative reaction in proteolytic but positive in pectolytic assays was derived from the insertion of Tn5 into the *prt* gene. The loss of pectolytic activity in Pel mutants is accompanied by the loss of tissue-macerating ability. However, the loss of proteolytic activity in Prt mutants does not affect the tissue-macerating ability. The proper function of the *pel* gene is therefore absolutely required by the bacterium to induce soft-rot; proteases produced by the bacterium however do not seem to play any signigicant role in tissue-maceration. The Out mutant, characterized by producing reduced levels of enzymes in the cells, results presumably from the inactivation of *out* gene involved in the export of both Pel and Prt. Like the Pel mutants, the Out mutants are also unable to induce soft-rot although low levels of Pel activity have been detected (8). The Rep mutants are especially interesting because they simultaneously lose the ability to produce Pel and Prt, and to form mucoid colonies in some strains. We now have evidence that the Rep mutants were derived from the insertion of Tn5 into a regulatory gene *rep* which controls production of polymeric molecules located outside of the cells (9), a topic to be discussed later.

CLONING AND CHARACTERIZATION OF PEL GENES

The gene encoding the Pel enzyme has been cloned from the genome of *P. viridiflava* strain SJ074 and efficiently expressed in *E. coli*. After a series of deletion subcloning and analysis by transposon mutagenesis, the *pel* gene was located in a 1.2-kb *Pst*I-*Bgl*II genomic fragment. This fragment appears to contain a promoter at the *Pst*I end required for self-expression. The cloned *pel* gene is capable of restoring the Pel-producing and the disease-causing ability in Pel-negative mutants previously isolated by Tn5 mutagenesis (11). By using the cloned *pel* DNA as a probe, we were able to detect *pel* homologs in a number of bacterial pathogens which normally did not produce Pels in culture (6,11). The nucleotide sequence of this gene has been partially determined. DNA sequence data shows that this *pel* contains a putative signal peptide at the N-terminus and exhibits very little homology with the Pels of *Erwinia* (1; Fig.2) The gene encoding the alkaline Pel of *P. fluorescens* has also been cloned, and located in a 1.7-kb of *Sal*I-*Xho*I genomic fragment (6). Nucleotide sequence analysis of this gene reveals an open reading frame of 1140 bp. The predicted Pel protein consists of 380 amino acid (aa) residues and shares approximately 45% homology with the PelE gene of *E. chrysanthemi* EC16. A 29 aa sequence located at the N-terminus of the Pel-coding region possesses some features of typical signal peptides that are required for the transport of proteins across the inner membrane. A hydrophobic region and a consensus signal peptidase recognition sequence (Ala-X-Ala) have been identified. The potential ribosome-binding site (AAGGA) is situated 11 bases upstream of the translational start codon (ATG). Furthermore, a potential Rho-

independent transcriptional termination signal (*CCCTCGGTGACGAGGG*) was identified 29 bases downstream of the translational stop codon (TAA) (2; and Fig.3). To determine if the leading 29 aa of the predicted Pel-coding sequence indeed function as a signal peptide, these sequences were fused to a DNA fragment encoding the mature cucumber chitinase gene. After being introduced into *E. coli*, this construct was able to direct the synthesis of high levels of cucumber chitinase in the bacterium (2; and Fig.4).

PHYSIOLOGICAL REGULATION OF PEL PRODUCTION

At present, very little is known about the biochemical mechanism governing Pel production in soft-rotting pseudomonads. In the majority of *P. fluorescens* strains, Pel production is induced by pectic substrates. However, in some strains, Pel production is not affected by the type of carbon sources included in the medium. Recently, we have examined the mode of Pel production in 24 strains of *P. fluorescens* (10). We found that Pel production in four out of 24 strains was not induced by pectic substrates but by calcium. When grown in glycerol-containing media, these four strains produced ten times more Pel in the medium supplemented with 1 mM calcium-chloride than in that without the calcium-chloride supplement. Furthermore, the Pel produced by bacteria grown in the calcium-containing medium was largely excreted into culture fluids; whereas the Pel produced by bacteria grown in calcium-deficient medium was retained within the cells. Calcium is thus required not only for the synthesis but also for the export of Pel. The optimal calcium concentration required for Pel production was determined to be 0.2 mM. A linear correlation was observed between the amounts of Pel produced and the concentrations of calcium included in the medium. Moreover, the calcium-requirement for Pel induction could not be replaced by other divalent metal ions such as Zn, Fe, Mn, and Mg. These results indicate that the effect of calcium on Pel production is concentration-dependent and cation-specific.

In addition to its role in regulating Pel production, calcium is also required for the catalytic action of the enzyme (15). Since most strains of *P. fluorescens* do not produce polygalacturonase, calcium is required by the Pel to initiate the first step of pectin degradation. The calcium availability would then determine if *P. fluorescens* could successfully infect plants. Based on this reasoning, we suggested that one possible approach to control *Pseudomonas* rot is to limit the availability of calcium to infecting bacteria in plant tissues. We have assessed this possibility by treating potato tuber disks with the calcium-chelator EDTA (10). The EDTA exhibited bacteriocidal activity against *Pseudomonas* at a minimal concentration of 4 mM. When potato tuber disks were treated with EDTA at a concentration of 0.13 mM (approximately 40 ppm), the development of *Pseudomonas* rot in potato tuber disks was totally inhibited. These results show the possibility of using EDTA as a disease control agent.

MOLECULAR GENETIC REGULATION OF PEL PRODUCTION

As mentioned earlier, by using Tn5 mutagenesis, we have identified a group of *P. viridiflava* mutants which simultaneously lose the ability to produce Pel, Prt, and exopolysaccharide. When the genomes of these four mutants designated as Rep were probed with Tn5, each mutant was found to have Tn5 inserted in one of two *Eco*RI fragments. These two *Eco*RI genomic fragments were assumed to contain two distinct loci *rep*A and *rep*B involved in the regulation of Pel, Prt and exopolysaccharide production (9). A cosmid clone carrying the putative *rep*A locus has been identified in the library constructed from the wild-type strain of *P. viridiflava*. Complementation studies show that this clone is capable of restoring the ability of some Rep mutants to produce Pel, Prt, and to form mucoid colonies. After a series of analyses with restriction enzymes and transposon mutagenesis, the region containing the putative *rep*A gene was located in a 3.2-kb subfragment of pLAI31. Recently, a clone carrying the putative *rep*B gene has been identified. The exact mechanism by which *rep* genes regulate extracellular enzymes and exopolysaccharide production is not clear. We suspect that the *rep* gene may encode a positive activator protein. An interaction between this Rep protein and the *cis*-acting element (possibily present in *pel*, *prt* and *alg* genes) is required to activate gene expression of the enzymes and exopolysaccharide synthases (Fig. 5).

BIOCHEMICAL CHARACTERIZATION OF PROTEASES

Unlike the Pel, the Prt produced by *P. fluorescens* and by *P. viridiflava* appears to exist in at least two forms with pIs ranging from 5.9 to 7.6 (12). The Prts produced by *P. fluorescens* are resistant to heat treatment and dependent on Zn for activity. Moreover, the presence of calcium in the culture media is absolutely required by the bacterium to produce the active form of the enzyme. Although the Prts produced by both *P. fluorescens* and *P. viridiflava* do not seem to play a significant role in maceration of plant tissues, they are capable of causing spoilage (gelation) of raw milk. This result indicates that pectolytic fluorescent pseudomomads which normally cause soft-rot of fresh fruits and vegetables may also have the potential to cause problems during production of raw milk. Recently, the gene encoding Prt enzyme has been cloned from the genome of a *P. fluorescens* strain. The Prt gene of *P. fluorescens* was expressed only poorly in *E. coli*. It is presently unclear as to why *Pseudomonas* genes usually express very poorly in *E. coli*. One possibility is that the promoter of *Pseudomonas* gene such as *pel* and *prt* does not follow the typical -10/-35 concensus pattern of *E. coli* promoters. Since the synthesis and export of both Prt and Pel enzymes are under the control of the same set of *out* and *rep* genes, investigations are underway to determine how a change in the molecular structure of *prt* and *pel* genes may affect the proper function of *out* and *rep*.

CONCLUDING REMARKS

By use of molecular genetic and recombinant DNA technologies, we have identified, cloned and characterized a set of disease-related genes from the soft-rotting bacteria *P. fluorescens* and *P. viridiflava*. All of these genes (*pel, prt, out,* and *rep*) are involved in the production or secretion of extracellular enzymes (Pel and Prt) and exopolysaccharide (Alg). We have provided genetic and biochemical data demonstrating that production of Pel is absolutely required by the bacteria to induce soft-rot in plants. However, the exact mechanism of how the *out* and *rep* genes controls secretion and synthesis of Pel remains obscure and needs to be further investigated. We are currently very interested in knowing if *rep* genes control production of other biomolecules in addition to Pel, Prt, and Alg. A better understanding of the Rep function may provide a new means for us to genetically improve the effectiveness of *Pseudomonas* strains as disease-control agents (5) and to increase the yield of industrially-important proteins produced by certain members of *Pseudomonas* species.

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